



Docket No. 55591 RCE (71699)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 CFR 1.131

The undersigned declare as follows:

1. We are co-inventors of the above-identified patent application assigned to The Johns Hopkins University.
2. Prior to September 1998, we conceived of and then diligently reduced to practice the methods of introducing into endothelial cells of an autologous vein grafts from a mammal an effective amount of at least one nucleic acid encoding thrombomodulin (TM), NF- κ B inhibitor, or a functional fragment of TM; provided that when the agent is thrombomodulin, the nucleic acid further encodes the NF- κ B inhibitor, wherein the introducing is performed *ex vivo* or by direct injection into the graft, and transplanting the vein graft into the mammal as disclosed and claimed in the above-identified patent application.
3. We diligently worked to reduce the methods to practice until the filing of the provisional application on May 22, 2000.
5. Attached as Exhibit 1, Figures 1 – 20, are true and accurate copies of laboratory notebook records with dates deleted. The notebook records demonstrate the conception, reduction to practice and diligence from conception to the filing of the application. The exhibits show that the constructs for the expression were received prior to September 1998 representing proof of the conception of the methods of using thrombomodulin to prevent early vein graft thrombosis as described in paragraph 2 above (Figure 1).

6. Figure 2 shows successful adenovirus-mediated gene transfer of and expression of B-galactosidase marker genes in rabbit vein grafts. Figure 3 demonstrates that we were also transducing rabbit vein graft with the adenovirus vector expressing human TM (AdTMh5; Figure 3). Figure 4 demonstrates that we were able to quantify native TM expression in vein grafts by harvesting rabbit vein grafts at various time points. Figure 5 is a page from Dr. Antony Kim's notebook demonstrating TM expression over time (TMTC series of rabbits) outlining a visual scoring system devised to quantify native TM expression in rabbit vein grafts.

7. Figure 6, a page from Dr. Rade's notebook, depicts the continued generation of rabbits for TM quantification (C6W) and the transduction of rabbit vein grafts with control adenovirus (Adl312). Figure 7, a page from Dr. Kim's notebook, details a digital scoring system for TM expression in vein grafts. Figure 8, a log from Dr. Rade's notebook demonstrates transduction of a series of rabbit vein grafts with a different control adenovirus (AdRNull-1). Figures 9 and 10, from Dr. Kim's notebook, document TM expression in rabbit vein grafts using the digital imaging system.

8. Figure 11, a page from Dr. Rade's notebook, shows a series of rabbit vein grafts transduced with AdTMh5. Figures 12-14, from Dr. Kim's notebook, document the measurement of protein C activation and human TM protein expression in rabbit vein grafts transduced with either the AdTmH5 or control adenoviral vectors. Figure 15, a page from Dr. Richard Sohn's notebook, demonstrates the construction of an adenoviral vector expressing IkB, a potent NF- κ B inhibitor. Figures 16, a page from Dr. Richard Sohn's notebook, shows the measurement of NF- κ B activation in rabbit vein grafts.

9. Figure 17 documents the ability of the IkB adenovirus in preventing TM down-regulation in response to inflammatory cytokines. Figure 18, a page from Dr. Rade's notebook, details the group of rabbit vein grafts that were transduced with the IkB adenovirus. Figure 20 is

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a figure resulting from data demonstrating that over-expression of I κ B can reduce neointimal formation in rabbit vein grafts. Figure 21, a page from Dr. Sohn's notebook, shows that over-expression of I κ B also effectively inhibits NF- κ B activation in rabbit vein grafts.

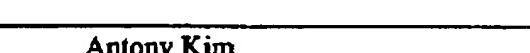
10. We hereby further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both (18 U.S.C. 1001), and that such willful false statements may jeopardize the validity of the above-identified application or any patent issued thereon.

Date: 10/30/06



Jeffrey J. Rade

Date: _____



Antony Kim

Date: 10/30/06



Richard Sohn

Rade et al.
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Page 3

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10. We hereby further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both (18 U.S.C. 1001), and that such willful false statements may jeopardize the validity of the above-identified application or any patent issued thereon.

Date: _____

Jeffrey J. Rade

Date: 2 Nov 2008


Antony Kim

Date: _____

Richard Sohn

Figure 1

Project No. 2

Book No.

TITLE *Cloning of human Thrombomodulin into AdLOX*

On Page No. _____

*Purpose: To make recombinant Adenovirus c human Thrombomodulin**1. Obtained plasmid clone of human Tm from ATCC (ATCC 61348) F*ATCC 61348, 61349 - plasmid clone of human thrombomodulin <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=pubmed&term=61348>Your query was:
thrombomodulin

Conf # S044066

\$105 + \$17.75 shipping

ATCC 61348, 61349 - plasmid clone of human thrombomodulin

ATCC 61348, 61349 - plasmid clone of human thrombomodulin, THBD

NAME: puc19TM15 (GDB:168893) [lambda:TM15]

DATABASE ACCESSION: DNA Seq. Acc.: M16552

VECTOR: plasmid vector: pUC19

ORGANISM: THBD, Homo sapiens (human)

TISSUE: umbilical vein endothelial cells

GENE NAME: in insert THBD, thrombomodulin (GDB:119613)

CHROMOSOME: THBD 20 p11.2

DNA: THBD cDNA

CONSTRUCTION: Insert lengths (kb): THBD 3.70

Excise by: EcoRI or Sall

6.40

Sequence Position: DNA Seq. Acc.: M16552

MARKERS: ampR

DEPOSITORS: J. E. Sadler

REFERENCES: Biochemistry 26: 4350-4357, 1987

J. Biol. Chem. 264: 20705-20713, 1989 (CIT:381458)

Genomics 5: 649-650, 1989 (CIT:13744)

DESCRIPTION: Restriction digests of the clone give the following sizes

(kb): EcoRI- 3.7, 2.7; SacI- 6.5; Aval- 3.4, 2.0, 0.2; XbaI- 5.9,

0.54, 0.2, 0.1. (ATCC staff)

There is 64% homology between this probe and bovine thrombomodulin. A single band of 3.7 kb is detected in human placenta and endothelial cell poly(A)+ RNA. The insert includes 146 nt of 5'-noncoding sequence, an open reading frame of 1725 nt and 1779 nt of 3'-noncoding sequence including 40 nt of poly(A) tail. The insert contains the following sites separated by (bp) (approx): EcoRI- 270- SmaI- 160- PstI- 240- PstI- 210- PstI- 100- PstI- 530- KpnI- 310- PstI- 1080- HindIII- 110- BamHI- 205- PstI- 475- EcoRI. (Biochemistry 26: 4350-4357, 1987)

GROWTH CONDITIONS: Medium 1227 37C

SHIPPED: 61348: freeze-dried Escherichia coli SURE

61349: dried purified DNA (200 ng)

PRICE CODE: 61348 D -- TIGR/ATCC SPECIAL COLLECTION OF HUMAN cDNA

CLONES

61349 D -- TIGR/ATCC SPECIAL COLLECTION OF HUMAN cDNA CLONES

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Figure 2

54

Project No.

Book No.

TITLE : TS OF KARMA - MULGARH

From Page No. 60

Purpose: STRA WIL GRAFT SEEDLING X GAE, PLANT: JUNGLE
340 FARMING IN 100% GROWTH WITH OPEN
CONSTRUCTION & PHOTOGRAPHIC NIGHT CHART.

① BGAV-

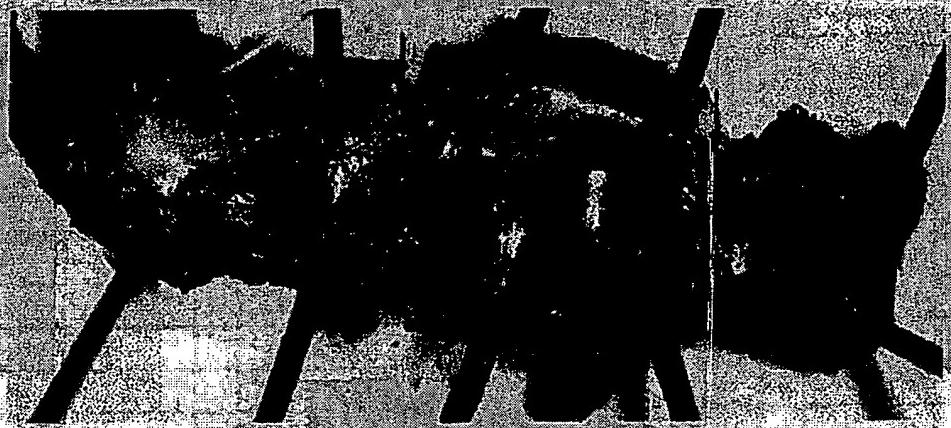
united

→ unroot

TC = 200.00 m-199 + Ad. CHARGE (50%)

300.00 PESU

→ 400.00 PESU (300.00 PESU + 100)



BEST SIGHT = MDP CANON

AMOUNT

② 13BAU-4

→

Same unroot

unroot



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Date

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Figure 3

Day 300
TMI

1 T views in 1025
not in views at 1135

Ad-Techs

Clamp 1143
Clamp off 1238

Pushing and clamp 11
Collet good - no blow or leak 1143

good leak still there in 1143

Clamp 11 removes huge rent in proctus

soft

Repaired as best as we know
still patent

One finger push to a depth of 10 mm

discovered

Signet leak & dash now.

>>> still see

Please

Signet patent fully - left open

HIP

75 X

P. No.
(
Pleas
Sect
8 gr
Gia
Sue
Please

Figure 4

Figure 5

TITLE	Rabbit Vein Graft Analysis (cont'd)										Project No.																																																							
From Page No.											Book No.																																																							
grading system (cont'd)																																																																		
classification of thrombomodulin expression (intensity)																																																																		
<table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 15%;">class</th> <th colspan="10" style="text-align: center;"><u>description</u></th> </tr> </thead> <tbody> <tr> <td>1+</td> <td colspan="10">trace, minimal</td> </tr> <tr> <td>2+</td> <td colspan="10">patchy, scattered</td> </tr> <tr> <td>3+</td> <td colspan="10">confluent, but not maximal intensity</td> </tr> <tr> <td>4+</td> <td colspan="10">maximum intensity</td> </tr> </tbody> </table>												class	<u>description</u>										1+	trace, minimal										2+	patchy, scattered										3+	confluent, but not maximal intensity										4+	maximum intensity									
class	<u>description</u>																																																																	
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4+	maximum intensity																																																																	
<u>CIRC.</u>		<u>day1</u>		<u>INTENS.</u>		<u>day1</u>		<u>Raw Data</u> \bar{x} <u>Mean</u> + <u>S</u> (compiled by Peiker)																																																										
		2	2	2	2	2	2																																																											
		3	3	2	2	2	2																																																											
		3	3	2	2	2	2																																																											
avg		2.666667		avg		2.166667																																																												
STD		0.57735		STD		0.235702																																																												
<u>day3</u>		<u>day3</u>		<u>day3</u>		<u>day28</u>		<u>day28</u>																																																										
		2	2	02/01	1.5	4	4							02/01																																																				
		2	2	1	1	3	3																																																											
		2	2	1.25	1.25	3	3																																																											
avg		2		avg		0.25																																																												
STD		0		STD		0.235702																																																												
<u>day5</u>		<u>day5</u>		<u>day5</u>		<u>control</u>		<u>control</u>																																																										
		3	3	2	2	4	4							03/04																																																				
		2	2	2	2	3	3																																																											
		2.5	2.5	2	2	3.25	3.25																																																											
avg		2.5		avg		0.381881																																																												
STD		0.408248		STD		0																																																												
<u>day7</u>		<u>day7</u>		<u>day7</u>		<u>avg</u>		<u>avg</u>																																																										
		2	2	2	2	4	4							03/04																																																				
		3	3	2.5	2.5	4	4																																																											
		3	3	2	2	4	4																																																											
avg		2.833333		avg		0																																																												
STD		0.235702		STD <td colspan="2">0.235702</td> <td colspan="6"></td>		0.235702																																																												
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<u>JM</u>																																																																		
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From Page No. 84

GUARDS SENT TO APIBSoldiers

285 C-30, LT-13
 285 C-33, LT-14
 285 C-34, LT-15

Soldier ID#sGuard ID#s

Adcon-1 2858

Adcon-2 2855

Adcon-3 2860

Adcon-4 2861

Adcon-5 2862

Adcon-6 - 2863

Adcon-7 2863

TMTC-23 3861

TMTC-24 3865

TMTC-21 3862

TMTC-22 3861

Adcon-8 3205

Adcon-9 3206

TMTC-25 3863

CGW-2 3208

CGW-3 3209

CGW-5 3210

CGW-8 3211

CGW-10 3212

SNT TO APIB

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Figure 6

VA 3

281

284

280

280

VO 16

φ

φ

φ

φ

280

7.5 x 10⁹

1100

Md

3c1

φ

φ

Mol

140

φ

φ

q

q

281

7.5 x 10⁹

1100

Md

φ

Gwa

Gwa

Gwa

Gwa

Gwa

1100

1100

1100

1100

1100

1100

1100

1100

1100

1100

1100

1100

1100

1100

Figure 7

104

Project No. _____

Book No. _____

TITLE TM in RVG model

From Page No. _____

Digital imaging of Thrombomodulin in RVG's !

Protocol

Hypothesis #1: TM protein expression decreases ^{in RVG} over time when ^{grafted into} ~~expressed in~~ ^{arterial circulation}.

Reason :

Hypothesis #2 : TM protein production shifts the thromboreistant balance of normal vasculature to cause a more fibrillar thrombotic state.

Reason :

Hypothesis #3 : The prothrombotic state left by the reduction of TM is a factor in VG failure

Reason :

Adobe

- ① shoot images via DMC
- ② adjust brightness in order to control for all vessels
- ③ mask the vessel \sim from endothelium

Sigma Scan

- ④ measurement setting = # of pixels (area) calibration 333 to 1000
- ⑤ measure # of pixels on vessel endothelium (choose orange for signal intensity threshold)
- ⑥ measure circumference of endothelium
- ⑦ record values including # pixels, circumference, & P/C

Quattro

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To Page No.

GUAFS SUT TO AP.P

Project No.
Book No.

Figure 8

Guaf 92

Specimen

Sunay

Hawas

DAYS

28

Virus

Adenovir.

Doser

2.5 MO?

346

Adeno-10

Adeno-11

Adeno-12

Adeno-13

Adeno-14

Adeno-15

Adeno-16

Adeno-17

C6W-13

C6W-14

C6W-15

C6W-16

C6W-17

Start

Frozn sections, GRAFT, ANEST, control int. v/v

Guaf

φ

3469

3468

346

346

346

346

1d

φ

φ

1°

Guaf

7d

φ

φ

3d

φ

φ

1d

φ

φ

3d

φ

φ

2d

φ

φ

GUAF CLOTHS

control int. v/v only
GUAF int. v/v

GRAFT only

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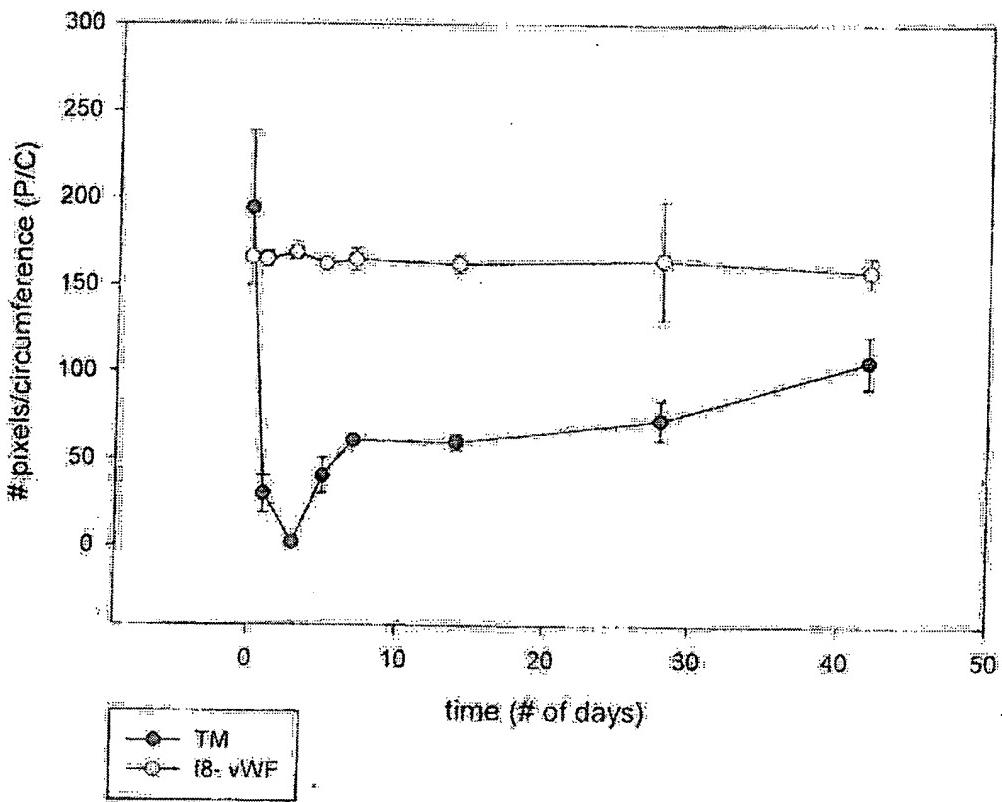
TM & F8-VWF

Project No.

Book No.

Figure 9

RVG Analysis



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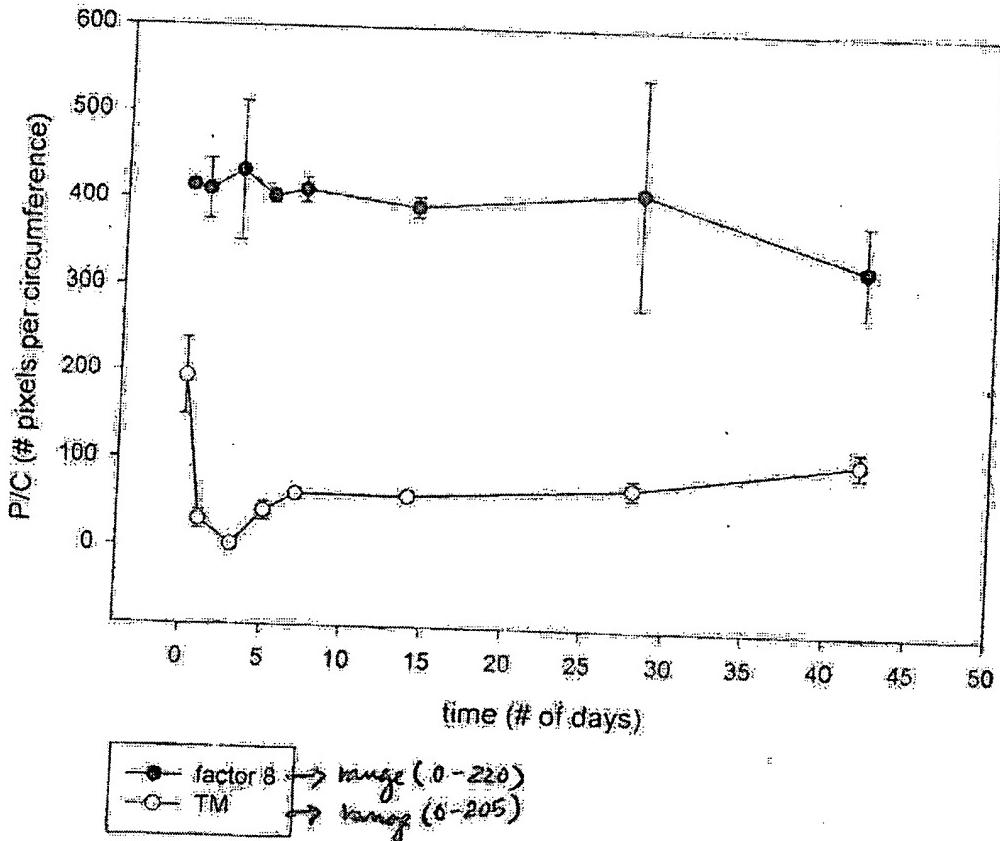
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TM & Factor 8 stains

TM and F8



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Date

Figure 11

Project No.	Book No.	TITLE: GRANTS SURG TO API'S			
100.					
From Page No.					
		SURG	Site	Vines	Waste
		Purfusion Fixus 10% Formal → 70% OTOH	For Invertibility		
4017	Adm H/S #3				
4018	Adm H/S #4				
4019	Adm H/S #5				
4020	Adm H/S #6				
4021	Adm H/S #8				
		SURG			
		Purfusion Fixus 10% Formal → 70% OTOH	For Invertibility		
4222	Adm H/S #12				
4233	Adm H/S #15				
4234	Adm H/S #16				
		HARD CAVES IN MPV			
4508	C-89				
4509	C-90				
4510	Adcon 33				
4511	Adcon 36				
		MPV Fixus 10% Formal → 70% OTOH	Admixer	3.35×10^6 PR/L	
		SURG			
		Purfusion Fixus 10% Formal → 70% OTOH	Admixer	3.35×10^6 PR/L	
4470	Adm H/S #14				
4481	Adcon 31				
4482	C-74				
4483	C-81				
		Purfusion Fixus 10% Formal → 70% OTOH	Admixer	3.35×10^6 PR/L	
		SURG			
		Purfusion Fixus 10% Formal → 70% OTOH	Admixer	3.35×10^6 PR/L	
4527	Adcon 35				
4528	Adcon 41				
4529	C-91				
4530	C-92				
4531	C-93				
		MPV Fixus 10% Formal → 70% OTOH	Admixer	3.35×10^6 PR/L	
		SURG			
		MPV Fixus 10% Formal → 70% OTOH	Admixer	3.35×10^6 PR/L	
Witnessed & Understood by me:		Date	To Page No.		

Figure 12

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Book No. _____

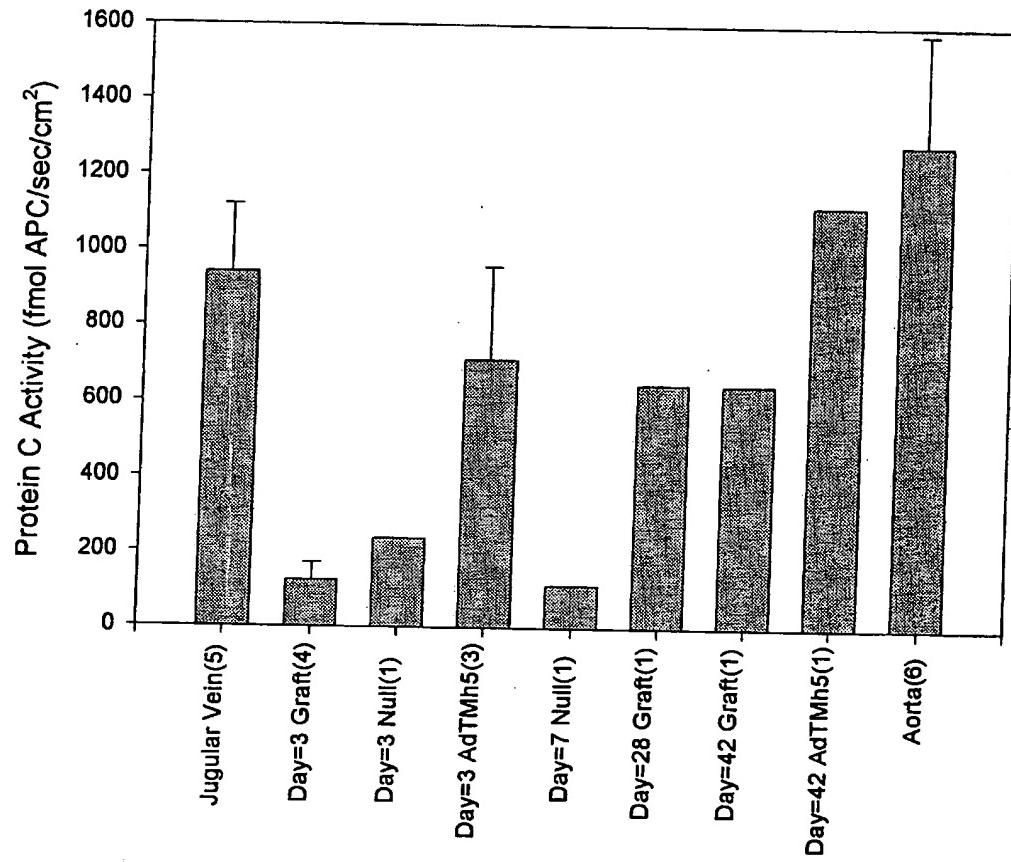
TITLE

PC Assay + Th Act. Assay

From Page No.

Thurs.

Protein C Assay (graph)



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Recorded by

Fri

① PC Activation Assay :

Figure 13

PC Activation Assay AdTMh5 #25 (day 7)

[thrombin] nM 10

[PC] uM 1

RVG (7 day) blank

AdTMh5#25	522	16.29
AdTMh5#25 - blank	505.71	

avg.	505.71	16.29
aPC	0.468275065	
aPC(fmoles/min/cm ²)	2498.2474717	

aPC Standard Curve

uM aPC mOD/min

Regression Output:

0	0.857	Constant	0.001813143
0.05	55.4	Std Err of Y Est	0.006520627
0.1	107.4	R Squared	0.998984198
0.2	213.8	No. of Observations	7
0.3	311.6	Degrees of Freedom	5
0.4	428.4		
0.5	549.2	X Coefficient(s)	0.00092239
		Std Err of Coef.	1.31539E-05

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Now TM ELISA for AdTMh5 + AdNull (American diagnostics inc kit #83)

Figure 14

THROMBOMODULIN ELISA

	<u>sample</u>	<u>OD (A450)</u>	<u>Dilution factor</u>	<u>TM</u>	<u>corrected TM</u>	<u>TM/BSA</u>
<u>AdTMh5</u>						
<u>day 3</u>						
	TM9	1.21	20	3.20804	24.20000	39.18483
	TM11	1.23	50	3.27142	163.57088	185.85785
	TM23	1.359	50	3.68020	184.00989	108.11640
<u>day 7</u>						
	TM25	3.166	50	9.40629	470.31439	354.42972
	TM27	2.466	50	7.18810	359.40506	149.04811
<u>day 14</u>						
	TM19	0.681	10	1.53173	15.31726	9.36070
	TM20	0.781	10	1.84861	18.48609	7.40715
	TM21	0.791	10	1.88030	18.80298	9.96799
<u>day 28</u>						
	TM22	2.187	50	6.30400	315.19977	231.53710
	TM29	1.162	50	3.05594	152.79683	66.64825
	TM30	1.148	50	3.01157	150.57865	65.59129
<u>day 42</u>						
	TM18	0.437	10	0.75853	7.58529	11.80432
<u>AdNull</u>						
<u>day 3</u>						
	NULL23	0.187	2	0	0	0
	NULL28	0.16	2	0	0	0
	NULL43	0.204	2	0	0	0
<u>day 7</u>						
	NULL25	0.195	2	0	0	0
	NULL27	0.206	2	0	0	0
	NULL31	0.201	2	0	0	0
<u>day 14</u>						
	NULL34	0.258	2	0	0	0
	NULL37	0.193	2	0	0	0
	NULL40	0.149	2	0	0	0
<u>day 28</u>						
	NULL29	0.172	2	0	0	0
	NULL36	0.194	2	0	0	0
	NULL39	0.165	2	0	0	0
<u>day 42</u>						
	NULL19	0.181	2	0	0	0
	NULL22	0.197	2	0	0	0
	NULL24	0.188	2	0	0	0

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TITLE _____

Figure 15

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Primer "RSV1":

AGCACC GTG CATT GCT TGA

T6 insert present, then fragments \Rightarrow ~1300-1400 bp.

PCE: (1) (2)

RSV

WT

(3)

RSV

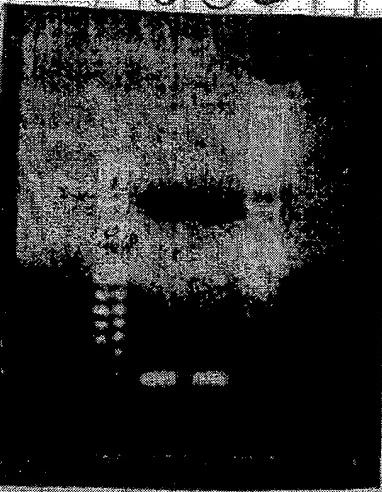
AS

pAdex R, 1KB wt

AS

Viral plate: overlaid
2nd layer MEM agar.

(1) (2) (3)



✓ No insert in viruses
 ✓ Ctrl (shuttle plasmid)
 ✓ verified PCE works

\Rightarrow Based out plates \Rightarrow for CMV, BS 1KB

(1) Pick up 6 plaques and inoculate 6 well-plate of 293 cells

(2) Thaw CEF 8 cells

(3) Digest [pAdex R 1KB wt] or [pAdex R 1KB AS] in hopes that recombination is 45% will be more efficient

Witnessed & Understood by me:

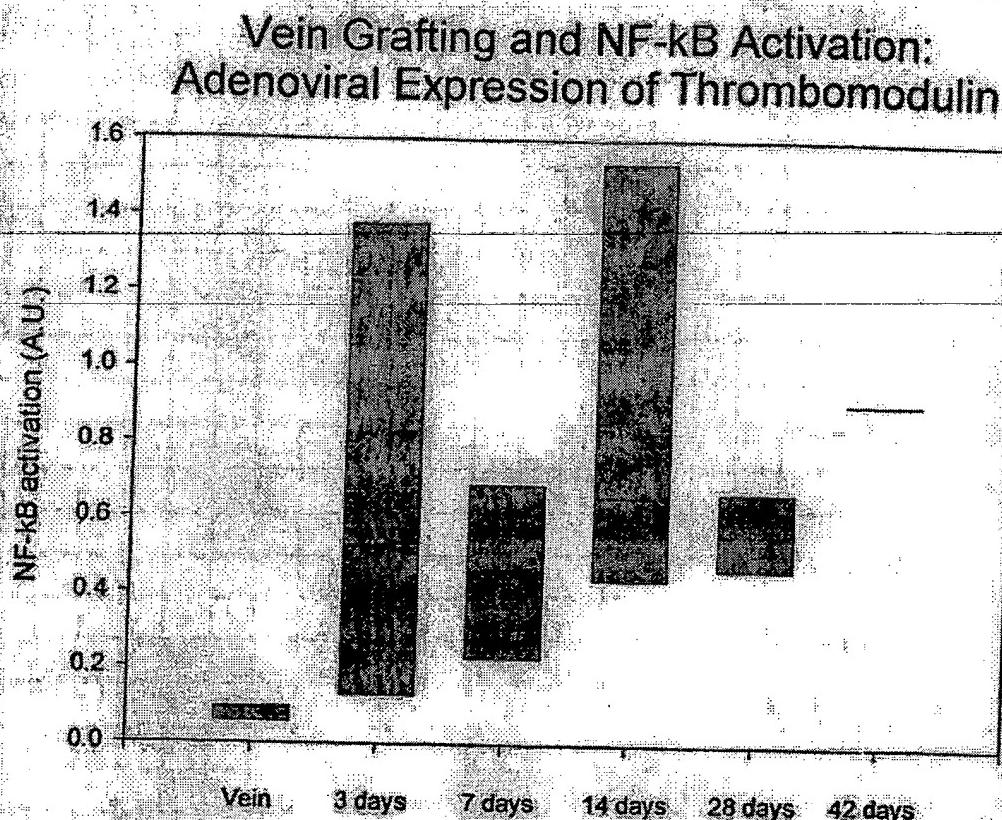
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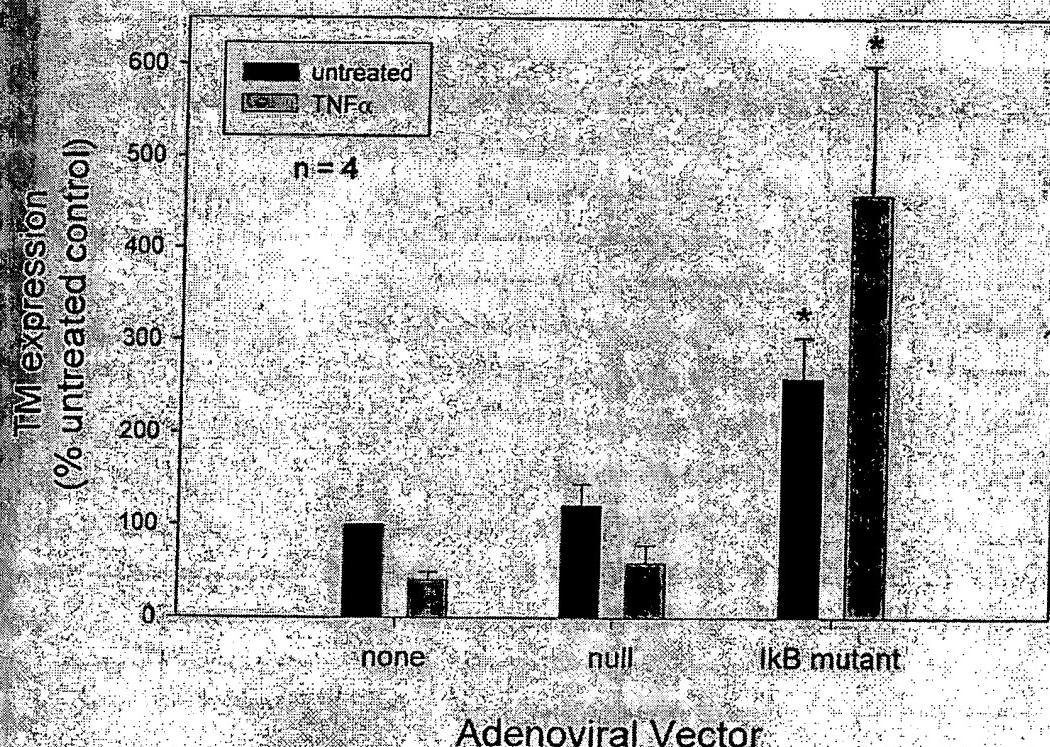
Plated 3.97 cells
 ↓
 3 days: 50% confluent
 ↓
 nearly confluent → infected 3 times
 ↓
 C6L C6R AdTM
 ↓
 90 min
 ↓
 Cells =
 Detached 2.5 ml
 ↓
 90 min
 ↓
 C6L C6R
 ↓
 100 Moi (5 μl)
 in 600 μl

Project No..
Book No..

Figure 17

	ctrl	TNF	null	null + TNF	IkBm	IkB + TNF
gel 1	598	274	519	148	1262	1290
gel 2	249	63	430	228	831	1826
gel 3	286	84	423	265	950	1918
gel 4	1088	668	841	288	1754	2330
as % ctrl	100	45.8194	86.7893	24.74916	211.0368	215.7191
	100	25.3012	172.6908	91.56627	333.7349	733.3333
	100	29.37063	147.9021	92.65734	332.1678	670.6294
	100	61.39706	77.29779	26.47059	161.2132	214.1544
mean	100	40.47207	121.17	58.86084	259.5382	458.459
S.D.	0	16.53125	46.46073	38.40392	87.17875	282.3589
S.E.M.	0	8.265623	23.23036	19.20196	43.58938	141.1794
t-test						
vs. control			0.397268		0.010579	
vs. null					0.031108	
vs. null-TNF						0.030975

Inhibiting NF- κ B Activation In HUVEC Upregulates TM and Prevents TM Downregulation By TNF α



* P < 0.05, compared to respective null-transduced

Recorded by

Figure 18

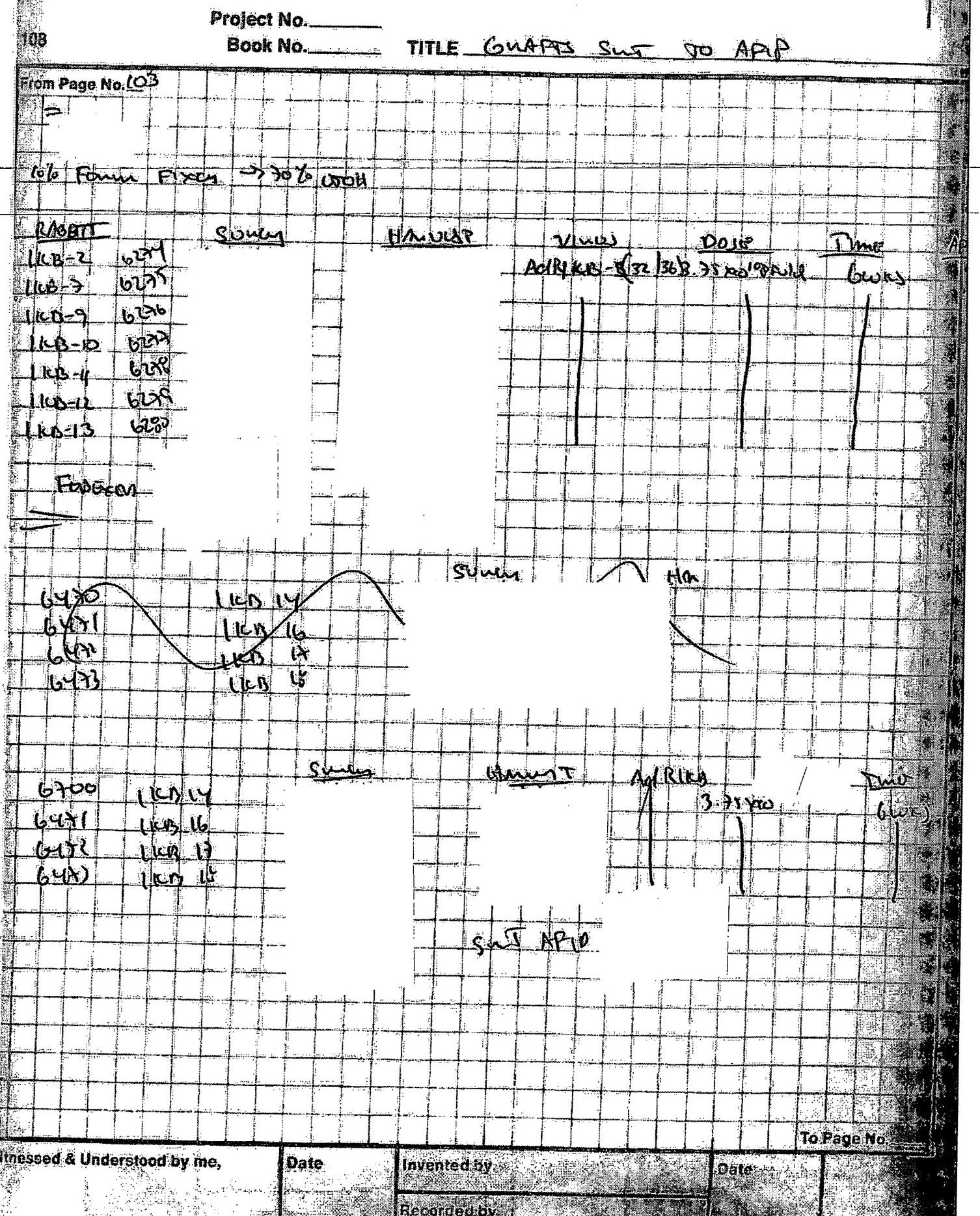


Figure 19

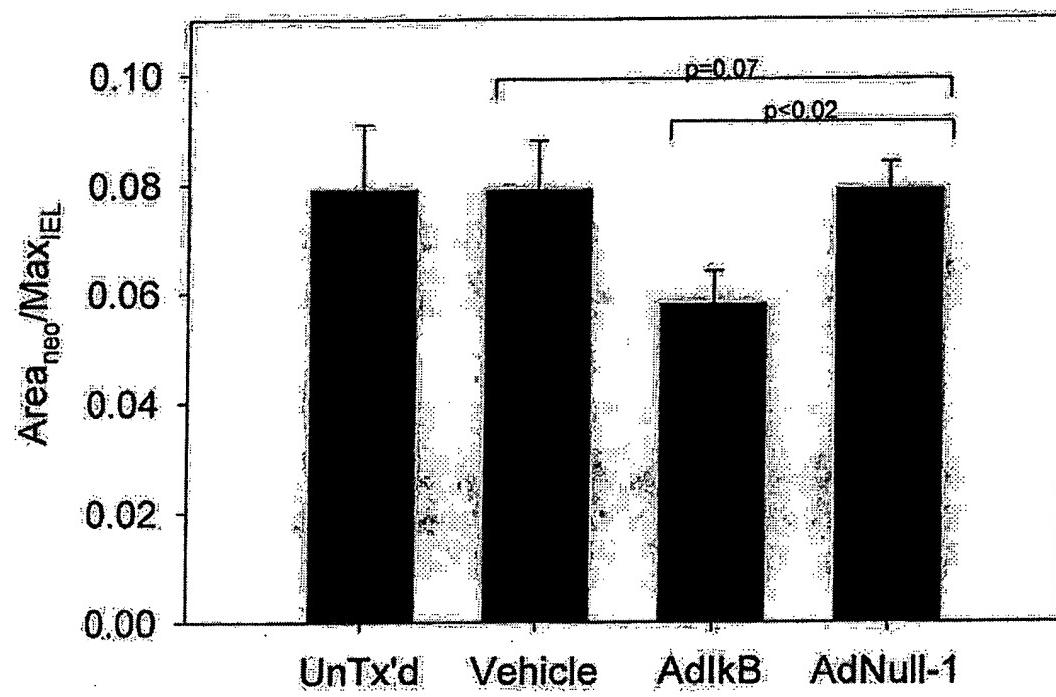
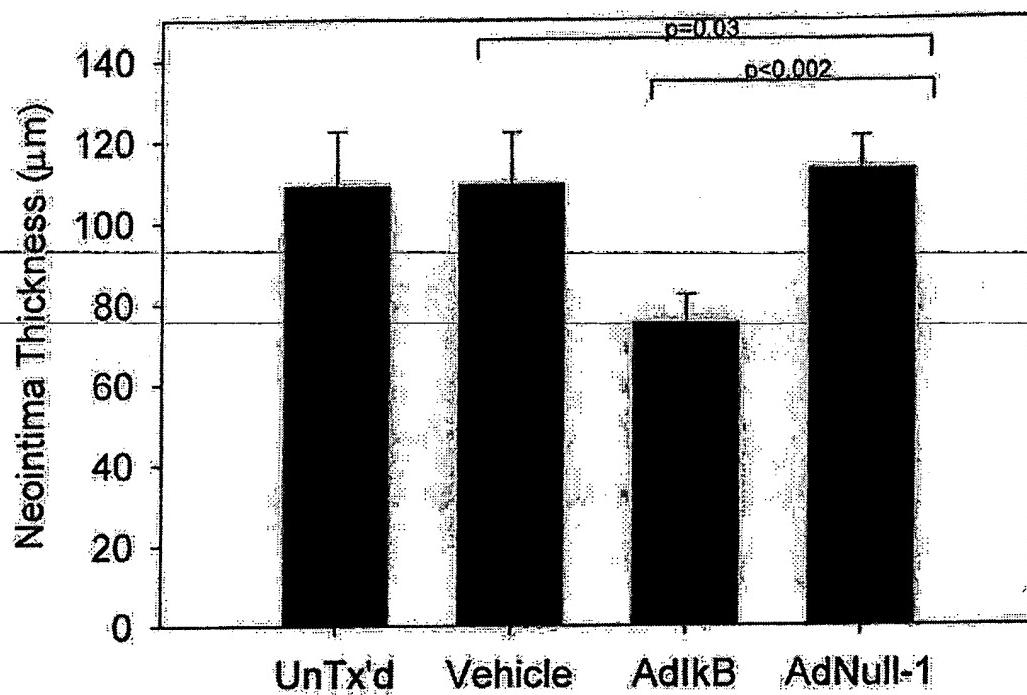
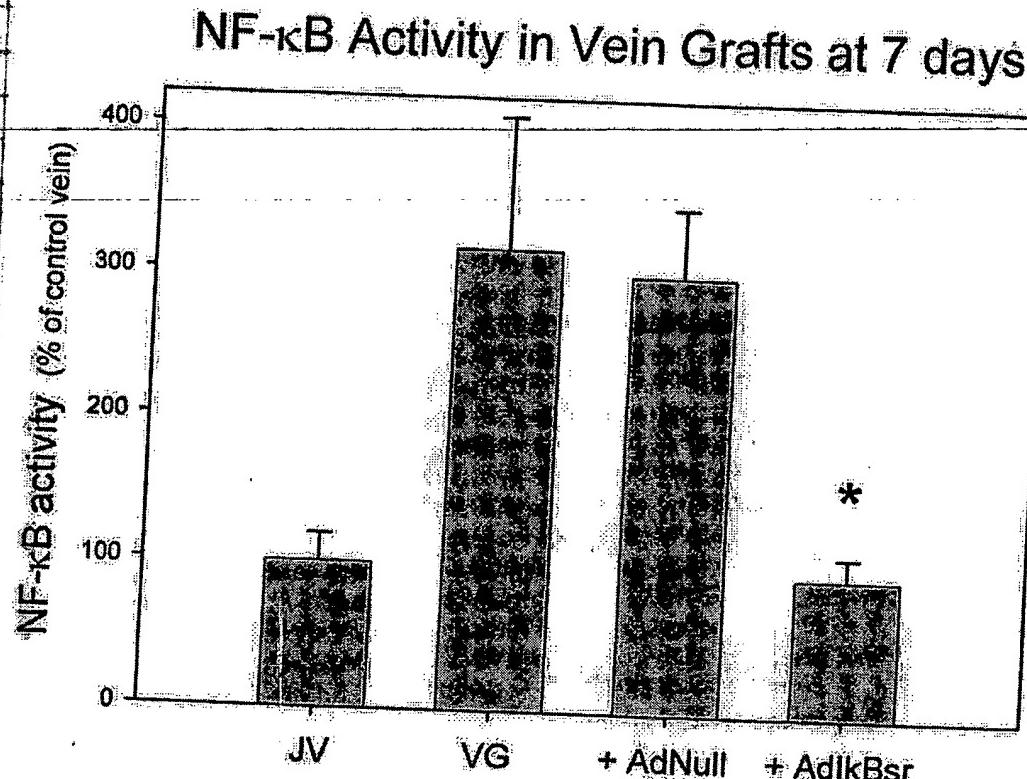


Figure 20



* P=0.014, as compared to AdNull-transduced vein graft.

Recombinant Transducing NUVEC = 7 days kit:

① Tfx PEI-RGD (Qbiogene Molecular Biology)

② Tf \times = 50 (Promega)

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